IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Confirmation No. 7075
Toshikazu NAKAMURA et al.	Attorney Docket No. 2006_0047A
Serial No. 10/565,301	Group Art Unit 1623
Filed : April 19, 2006	Examiner Jonathan S. Lau

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir:

I, Kazuhiro FUKUTA hereby declare that:

I was born in Mie prefecture, Japan, in 1966;

I am a citizen of Japan and a resident of 202 T.Mezon Lobelia, 18-27, Onoharahigashi 5-chome, Mino-shi, OSAKA 562-0031 JAPAN;

I received my doctor degree on the study of "Biosynthetic control of N-linked sugar chains in mammalian cells" at Kyoto University, Kyoto, Japan, in 2001;

I have worked as a scientist of Osaka University in Japan from 2002 until now and have engaged in a study on induction of hepatocyte growth factor;

I am one of the inventors for this application;

I have some reports relating to the activity of heparin or fragments thereof. The reports are as follows:

1. Sakiyama R, Fukuta K, Matsumoto K, Furukawa M, Takahashi Y, Nakamura T. 2007, J. Biochem. 141:653-660.

2. Fukuta K, Nakamura T. 2008, J. Pharm. Pharmacol. 60:499-503.

The experiments given below were conducted under my supervision.

Experiment 1

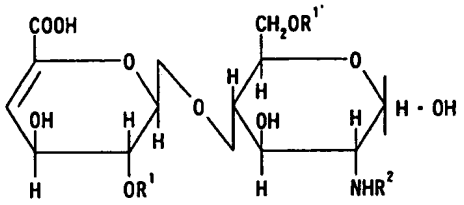
(1) Purpose

Experiment 1 was designed to determine the minimum heparin fragment which has an ability of promoting HGF production.

(2) Method

The activity to promote HGF production of the following disaccharide compounds sulfated at different positions were measured: 2-Sac-2S (Heparin disaccharide III-H; manufactured by Sigma), 2-Sac-6S (△DiHS-6S; manufactured by SEIKAGAKU CORPORATION) and 2-Sac-NS (△DiHS-NS; manufactured by SEIKAGAKU CORPORATION). The structural formulae of these compounds are shown in Table 1.

Table 1

Symbol	Simplified structure			
		R^1	R^2	$R^{1'}$
2-Sac-2S	ΔUA-2S-GlcN	SO_3H	H	H
2-Sac-6S	ΔUA-GlcNAc-6S	H	$COCH_3$	SO_3H
2-Sac-NS	ΔUA-GlcNS	H	SO_3H	H

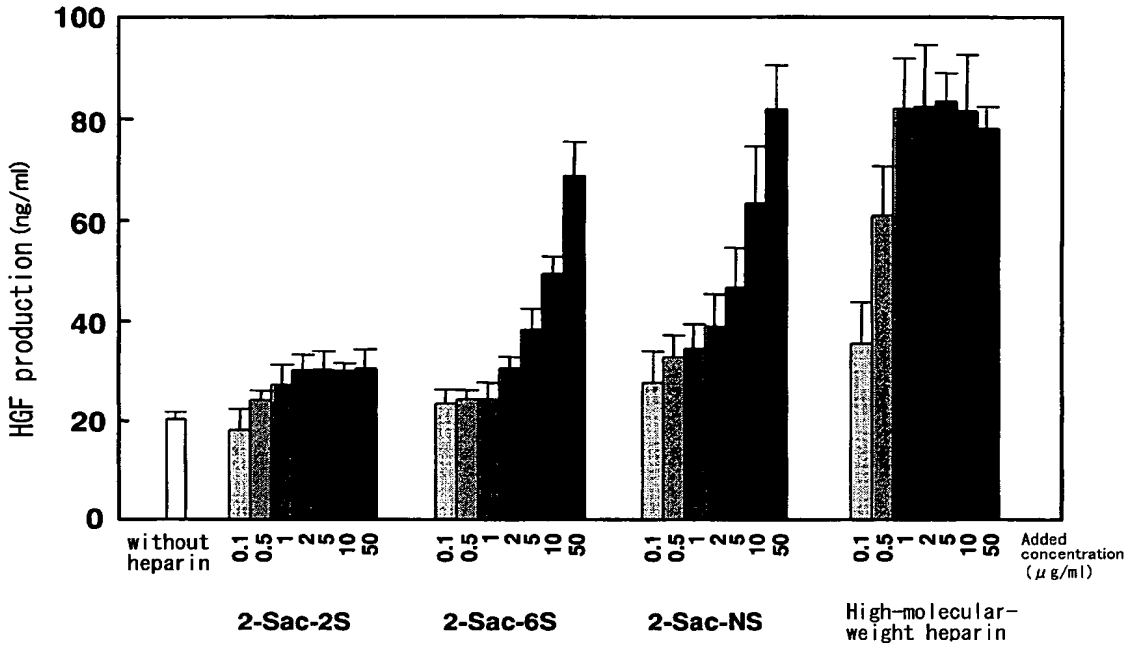
Activities to promote HGF production of heparin disaccharides fragments shown in Table 1 were measured by using MRC-9 cells, human fetal lung fibroblasts. The cells were cultured with the heparin disaccharide fragments and amounts of HGF produced by the cell were analyzed. In particular, first, MRC-9 cells were seeded onto a 48 well plate at a density of 5×10^4 cells/cm^2, and cultured in a DMEM medium containing 10% FCS for one day. After removing the medium, the cells were washed twice with 0.5 mL of PBS, and the medium was exchanged with a DMEM medium containing 1% FCS. At this time, the heparin disaccharide fragments were added to the medium at a final concentration ranging from 0.1 to 50 μg/mL. Thereafter, the culture was continued. After 24 hours, the culture supernatant was recovered, and an amount of HGF secreted into the culture medium was measured

by the ELISA method. An unfractionated high-molecular weight heparin was used as a positive control.

(3) Results

Results are shown in Fig.1 below.

Fig.1



HGF production by MRC-9 cells in the presence of heparin disaccharide fragments increased in a dose-dependent manner. The HGF production by MRC-9 cells in the presence of heparin disaccharide fragments was clearly larger than that in the absence of a heparin fragment.

(4) Conclusion

Since a heparin disaccharide fragment comprises an uronic acid residue and a glucosamine residue, the unit consisting of heparin fragment is a disaccharide fragment. Therefore, the results in Experiment 1 suggest that the minimum heparin fragment which has an ability of promoting HGF production is a heparin disaccharide fragment.

Experiment 2

(1) Purpose

Experiment 2 was designed to determine the maximum heparin fragment whose LPL releasing activity is suppressed.

(2) Method

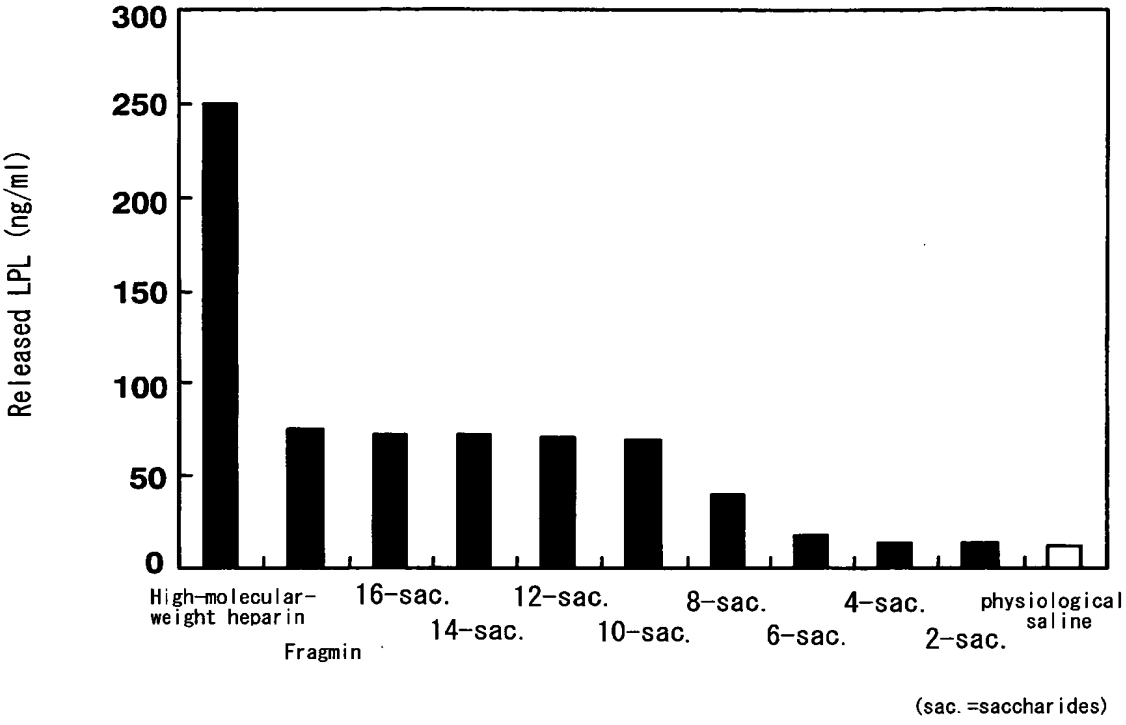
100 mg of high-molecular-weight heparin Na (Scientific Protein Laboratories Inc.) was dissolved in 2 mL of 50 mM sodium acetate buffer (containing 3 mM calcium acetate) (pH 7.0), and 0.5 unit of heparinase (SEIKAGAKU CORPORATION) was added to the mixture to react them at 37°C for 10 hours. The reaction mixture was heated at 100°C for 2 minutes to stop the reaction. 0.3 mL of the supernatant of the reaction mixture was subjected to a Superdex 30pg column (ϕ1.6 cm × 60 cm) (Amersham Bioscience) to perform gel filtration chromatography. As a mobile phase, 200 mM aqueous ammonium bicarbonate solution was used, and the solution was passed at a flow rate of 0.4 ml/min. Absorbance of the eluate at 230 nm was monitored, and the eluate was fractionated. Heparin fragments were separated and

recovered on the basis of molecular size. The heparin fragments were obtained as oligosaccharide fractions of disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, dodecasaccharides, tetradecasaccharides and hexadecasaccharides.

For measuring LPL releasing activity, these heparin fragments were administered to Wister rat (3-week old male) via the tail vein at 0.3 μg per 1 g of body weight and, after 10 minutes, blood was taken from axillary fossa venous plexus, and a LPL amount in the resulting plasma was measured by ELISA (Daiichi Pure Chemicals Co., Ltd).

(3) Results

Results of measurement of released LPL amount are shown in Fig. 2.



The plasma LPL concentrations in the case where heparin di to hexadeca-saccharides fragments were respectively administered to a Wister rat were clearly lower than that in the case where an undigested high-molecular weight heparin was administered to a Wister rat.

Furthermore, the plasma LPL level in the case where a heparin hexa-saccharides fragment was administered to a Wister rat was almost the same as that in the case where saline was administered to a Wister rat. And the plasma LPL level in the case where a heparin hexa-saccharides fragment was administered to a Wister rat was clearly

low than that in the case where a heparin octa-saccharides fragment was administered to a Wister rat.

(4)Conclusion

The results in Experiment 2 suggest that the maximum heparin fragment whose LPL releasing activity is suppressed is a heparin hexa-saccharides fragment.

Experiment 3

(1)Purpose

Experiment 3 was designed to determine whether or not heparin di to hexa-saccharides fragments have anti-blood coagulation activity.

(2)Method

Anti-blood coagulation activities of the heparin fragments obtained in Experiment 2 were assessed by measuring an activated partial thromboplastin time (APTT). APTT was measured using fresh plasma taken from Wister rat (male) and Data ϕ•APTT kit (Sysmex) which utilizes activation by ellagic acid. The heparin fragments were added to rat plasma at a concentration of 9 μg/mL or 3 μg/mL, and APTT values (second) were measured. Longer APTT value indicates higher anti-blood coagulation activity.

(3)Results

Results of APTT measurement are shown in Fig. 3.

Fig.3

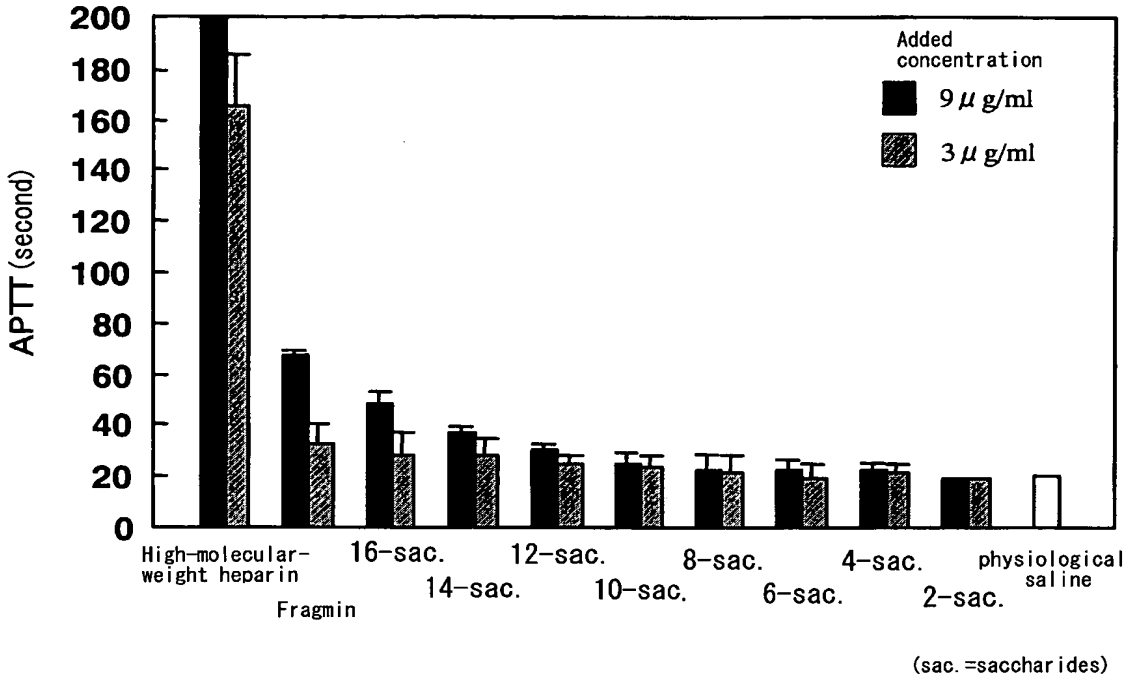


Fig. 3 indicates that APTT values in the cases where heparin di to deca-saccharides fragments were respectively mixed with rat plasma are almost the same as that in the case where saline was mixed with rat plasma.

(4)Conclusion

The results in Experiment 3 suggest that heparin di to hexa-saccharides fragments do not substantially have anti-blood coagulation activity.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: June 24, 2008

K. Fukuta

Kazuhiro FUKUTA